REMARKS

Claims 1-6 and 10-11 have been cancelled. Claims 7 and 9 have been amended. New claims 12-13 are added. Support for new claims 12 and 13 is found in the present specification at page 8, last paragraph and canceled claim 11. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Election/Restrictions

With this amendment, non-elected claims 1-6 are canceled. Applicants specifically reserve the right to file a divisional application to pursue non-elected inventions.

Priority

An English translation of a priority document is not necessary to claim priority, but only to overcome an intervening reference (see M.P.E.P. 201.15). As no intervening reference has been cited, it is not necessary for Applicants to provide an English translation of a priority document.

Applicants assert that the priority claim has been properly made by listing of the priority document on the Declaration/Power of Attorney document which was filed with the application and by submission of a certified copy of the foreign application (see M.P.E.P. 201.14(b)). As this application is the US National phase under 35 U.S.C. § 371, a copy of the certified priority document is conveyed by the International Bureau (PCT Rule 17.2(a)).

Rejection under 35 U.S.C. § 103(a)

Claims 7-11 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Ishida, et al. (EP 1147764.

This ground of rejection is addressed by amendment taken with the arguments below.

Claim 7 has been amended to recite administration of a compound of general formula (1) "to the skin of an individual in need thereof, whereby elongation of melanocytic dendrites is inhibited". The Federal Circuit in Jansen v. Rexall Sundown, Inc. (342 F.3d 1329 (Fed. Cir.

2003); copy provided as Attachment A) has ruled that the phrase "an individual in need thereof" must be accorded weight in determination of patentability.

In the present case, a person in need of inhibition of elongation of melanocytic dendrites is not necessarily the same person as a person in need of a combination of whitening effect, vitalizing the skin and preventing wrinkles as taught by Ishida, et al in paragraph 0006, cited in the Office Action.

The compounds represented by general formula (1) of the present invention inhibit elongation of melanocytic dendrites (see Example 1 of the present specification) and are effective for alleviating dyschromatosis on which melanin production inhibitors utilizing a tyrosinase inhibitory action are not effective or are less effective. See Examples 2 and 4 of the present specification. In contrast, Ishida, et al. teach compounds which have a whitening effect by inhibition of melanin production. Table 1 on page 8 of Ishida, et al. teaches compounds of Formulas II to V which inhibit melanin production.

Compounds of the present invention inhibit elongation of melanocytic dendrites and do not appreciably affect melanin synthesis or tyrosinase, TRP-1, TRP-2, and Pmel-17. In support, Applicants present Attachment B (Tada, et al. IFSCC Magazine, presented at 23rd IFSCC Congress 2004, Orlando FL). The paper reports on the effects of a number of botanical extracts on melanosomes and melanocytic dendrites, including centaureidin. While centaureidin was effective to shrink normal human melanocyte dendrites (Figure 2) and to inhibit melanosome transfer through dendrites (Figure 5), effects on melanin production were negligible (Figure 4).

Accordingly, a person in need of a skin treatment "whereby elongation of melanocytic dendrites is inhibited" would not turn to the compounds of the disclosure of Ishida, et al. because there is no indication in Ishida, et al. that the compounds disclosed by Ishida, et al. are useful to inhibit melanocytic dendrite elongation.

Furthermore, as stated by the Examiner in the Office Action, Ishida, et al. do not teach the compounds of the present application. While Ishida, et al. disclose a general formula (1) (page 3) which is generic to formula (1) of Applicants' claim 1, there is no hint that any of the compounds falling within the genus of formula (1) of Ishida, et al. are effective to inhibit melanocytic dendrite elongation. Ishida, et al. only teach inhibition of melanin production.

Claims 8, 9 and 13 are patentable for the reasons given above for claims 7 and 12 and for the following additional reasons. Claim 8 (and claim 9 as it depends from claim 8) is limited to centaureidin. The compounds of Ishida, et al., while structurally related to the compounds of Applicants, have an effect opposite to the effects of centaureidin. Namely, the compounds of Ishida, et al. inhibit melanin production while centaureidin has negligible effect on melanin production as discussed above with respect to Tada, et al. (Attachment B). Accordingly, claims 8 and 9 as it depends from claim 8 are clearly patentable over Ishida, et al.

Claim 9 is patentable over Ishida, et al. as claim 9 specifies the treatment of "dyschromatosis on which tyrosinase inhibitors have insufficient effect". As discussed in the Background section of the present specification, some forms of dyschromatosis cannot be effectively treated by inhibitors of melanin production such as tyrosinase inhibitors (see present specification, page 1, last line to page 2, line 13). Accordingly application of the method of Ishida, et al, in which inhibitors of melanin production are applied to skin, would not be satisfactory for treatment of forms of dyschromatosis in which inhibitors of melanin production are not satisfactorily effective. A person in need of a treatment for such forms of dyschromatosis would have no reason to apply the compositions disclosed by Ishida, et al. which are inhibitors of melanin production. Accordingly, claim 9 is clearly patentable over Ishida, et al.

The Office Action states that the claimed method is directed to "a new mechanism of action of the compounds of formula 1" (Office Action, page 5, last paragraph). However, as discussed above, the claimed method is directed to the treatment of a different condition than disclosed by Ishida, et al. Ishida, et al. is directed to treatment of conditions caused by melanin overproduction. Applicants' claimed invention is directed to treatment of conditions caused by elongation of melanocytic dendrites. While both treatments relate to skin, the two treatments are not interchangeable as some skin conditions result from elongation of melanocytic dendrites rather than melanin production.

The Examiner states that it would have been obvious to use Centaureidin for treatment of skin disorders because of similarity between Centaureidin and the structure of Formula I of Ishida, et al. (Office Action, page 7, first full paragraph). However, as presented above, Centaureidin clearly has different properties than the compounds of Table 1 on page 8 of Ishida, et al. While the compounds of Ishida, et al. inhibit melanin production, Centaureidin does not

appreciably inhibit melanin production as evidenced by Tada, et al. in Attachment B. Accordingly, Centaureidin is non-obvious over the compounds of Ishida, et al. Furthermore, Ishida, et al. are completely silent regarding a method to inhibit elongation of melanocytic dendrites. Accordingly, the claimed method is nonobvious over Ishida, et. al.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

Co-Pending Applications of Assignee

Further to the listing provided with the response to the Restriction Requirement submitted April 9, 2008, Applicant wishes to draw the Examiner's attention to the following co-pending applications of the present application's assignee.

Serial Number	Title	Filed
12/064001 (correction)	METHOD OF EVALUATING SKIN CONDITIONS AND METHOD OF ESTIMATING SKIN THICKNESS	15-Feb-2008
12/162977	SKIN-WHITENING COSMETIC	31-Jul-2008
12/162961	EXTERNAL PREPARATION FOR SKIN CONTAINING FLAVANONE DERIVATIVE	31-Jul-2008

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Oct. 27, 2008

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ATTACHMENT A

United States Court of Appeals for the Federal Circuit

03-1069

CHRISTIAN J. JANSEN, JR.,

Plaintiff-Appellant,

v.

REXALL SUNDOWN, INC.,

Defendant-Appellee.

John C. McNett, Woodard, Emhardt, Naughton, Moriarty & McNett, of Indianapolis, Indiana, argued for plaintiff-appellant. With him on the brief was Steve E. Zlatos.

Gary H. Levin, Woodcock Washburn LLP, of Philadelphia, Pennsylvania, argued for defendant-appellee. With him on the brief was Lynn B. Morreale.

Appealed from: United States District Court for the Southern District of Indiana

Judge John Daniel Tinder

United States Court of Appeals for the Federal Circuit

03-1069

CHRISTIAN J. JANSEN, JR.,

Plaintiff-Appellant,

v.

REXALL SUNDOWN, INC.,

Defendant-Appellee.

DECIDED: September 8, 2003

Before LOURIE, RADER, and SCHALL, Circuit Judges.

LOURIE, Circuit Judge.

Christian J. Jansen, Jr., appeals from the final decision of the United States District Court for the Southern District of Indiana granting summary judgment that Rexall Sundown, Inc. has not infringed Jansen's U.S. Patent 4,945,083. Jansen v. Rexall Sundown, Inc., No. IP 00-1495-C-T/G (S.D. Ind. Sept. 25, 2002). Because the court correctly construed the patent claims and correctly found no genuine issues of material fact on the question of infringement, we affirm.

BACKGROUND

Jansen is the sole inventor and owner of the '083 patent, which is directed to methods of "treating or preventing macrocytic-megaloblastic anemia" by administering a combination of folic acid and vitamin B_{12} "to a human in need thereof." '083 patent, col. 6, ll. 20-24, ll. 37-41. According to the patent, deficiencies of either folic acid or vitamin B_{12} can cause

macrocytic-megaloblastic anemia, also referred to as pernicious anemia, while a deficiency of vitamin B_{12} can also cause neurological problems. Id. at col. 4, ll. 13-25. When folic acid alone is utilized to treat macrocytic-megaloblastic anemia, the folic acid may mask a vitamin B_{12} deficiency. Id.; see also id. at col. 3, l. 65 – col. 4, l. 5. An objective of Jansen's invention is to administer both supplements together to avoid the masking problem. Id. at col. 4, ll. 25-48. The independent claims read as follows:

> 1. A method of treating or preventing macrocytic-megaloblastic anemia in humans which anemia is caused by either folic acid deficiency or by vitamin B_{12} deficiency which comprises administering a daily oral dosage of a vitamin preparation to a human in need thereof comprising at least about 0.5 mg. of vitamin B_{12} and at least about 0.5 mg. of folic acid.
>
> 4. A method of treating or preventing macrocytic-magaloblastic [sic] anemia in humans which anemia is caused by either folic acid deficiency or by vitamin B_{12} deficiency which comprises orally administering combined vitamin B_{12} and folic acid to a human in need thereof in sufficient amounts to achieve an oral administration of at least about 0.5 mg. of vitamin B_{12} and at least about 0.5 mg. of folic acid within one day.

Id. at col. 6, ll. 20-24, ll. 37-41 (emphases added).

The '083 patent is a seventh-generation continuation of a patent application filed in 1970. Every member of the '083 patent's lineage was abandoned in favor of the succeeding application until the '083 patent issued in 1990. Jansen's first application claimed the method as follows:

> A method of treating or preventing anemia in humans which comprises administering a daily oral dosage of a vitamin preparation containing at least .5 mg. of vitamin B_{12} and at least .5 mg. of folic acid, whereby anemia can safely be treated orally without determining whether it is caused by folic acid deficiency or by vitamin B_{12} deficiency.

In re Jansen, 187 USPQ 743, 744 (CCPA 1975). That original claim, while specifying approximately the same amounts of folic acid and vitamin B_{12}, does not specify the type of anemia being treated and says nothing about any need on the part of the human subject. The U.S. Patent and Trademark Office ("PTO") found that claim, as well as claims directed to the

composition of matter, to be obvious in light of prior art that taught administration of folic acid alone in the claimed range, vitamin B_{12} alone in the claimed range, and combinations of the two in smaller doses than claimed. The PTO found unpersuasive Jansen's argument that administration of both components in the higher, claimed doses was an unexpected solution to the masking problem, and the Court of Customs and Patent Appeals affirmed the PTO's rejections. Id. at 746. In his next five applications, Jansen persistently attempted to gain allowance of his claims in slightly different form, yet the PTO consistently rejected his attempts. In the prosecution of his seventh application, Jansen repeated his masking-avoidance argument and submitted an article that asserted that the medical community had come to realize the effectiveness of folic acid-vitamin B_{12} combination therapy to treat pernicious anemia only after Jansen's invention date. See William H. Crosby, Improvisation Revisited — Oral Cyanocobalamin Without Intrinsic Factor for Pernicious Anemia, 140 Arch. Intern. Med. 1582 (1980). The examiner agreed but noted that the claims, being directed to unspecified anemia, were not commensurate in scope with Jansen's showing of unexpected results. Jansen thereafter agreed to cancel his composition of matter claims and to narrow his method claims by requiring a specific type of anemia, viz., macrocytic-megaloblastic anemia, rather than anemia generally, and by adding to the claims the phrase "to a human in need thereof." The PTO then issued the '083 patent to Jansen.

Rexall markets to the general public an over-the-counter dietary supplement presently known as Folic Acid XTRA™ that contains folic acid and vitamin B_{12} within the claimed ranges. The Rexall product is labeled and advertised for maintenance of proper blood homocysteine levels, but not for prevention or treatment of macrocytic-megaloblastic anemia.

Jansen sued Rexall for inducement of and contributory infringement of the '083 patent. In the district court Jansen argued that all people are "human[s] in need" of "treat[ment] or prevent[ion] of macrocytic-megaloblastic anemia," but the court, without definitively construing

the "in need" phrase, rejected that argument. Jansen, slip op. at 14. Citing, inter alia, Rapoport v. Dement, 254 F.3d 1053 (Fed. Cir. 2001), the court then construed the phrase "treating or preventing macrocytic-megaloblastic anemia" to require that, in order to infringe the patent, the human subject of the claimed method take the compound with the intent of treating or preventing macrocytic-megaloblastic anemia. Jansen, slip op. at 16. Because the court found no evidence of such intent or purpose on the part of Rexall's customers, the court granted summary judgment of noninfringement. Id. at 16-17.

Jansen timely appealed to this court, and we have jurisdiction pursuant to 28 U.S.C. § 1295(a)(1).

DISCUSSION

Summary judgment is appropriate if "there is no genuine issue as to any material fact and . . . the moving party is entitled to a judgment as a matter of law." Fed. R. Civ. P. 56(c). "The evidence of the nonmovant is to be believed, and all justifiable inferences are to be drawn in his favor." Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 255 (1986). We review a district court's grant of a motion for summary judgment de novo. Ethicon Endo-Surgery, Inc. v. U.S. Surgical Corp., 149 F.3d 1309, 1315 (Fed. Cir. 1998).

A determination of patent infringement requires a two-step analysis. "First, the court determines the scope and meaning of the patent claims asserted . . . [Second,] the properly construed claims are compared to the allegedly infringing device." Cybor Corp. v. FAS Techs., Inc., 138 F.3d 1448, 1454 (Fed. Cir. 1998) (en banc) (citations omitted). Step one, claim construction, is an issue of law, Markman v. Westview Instruments, Inc., 52 F.3d 967, 970-71 (Fed. Cir. 1995) (en banc), aff'd, 517 U.S. 370 (1996), that we review de novo, Cybor, 138 F.3d at 1456. Step two, comparison of the claim to the accused device, requires a determination that every claim limitation or its equivalent is found in the accused device. Warner-Jenkinson Co. v. Hilton Davis Chem. Co., 520 U.S. 17, 29 (1997). Those

determinations are questions of fact. Bai v. L & L Wings, Inc., 160 F.3d 1350, 1353 (Fed. Cir. 1998).

On appeal, Jansen first argues that the court improperly construed the claims. More specifically, he contends that the court's construction improperly added to the claims an intent element, which is contrary to law as well as contrary to the ordinary meaning of the claim language, which does not suggest that the infringer's state of mind is relevant. Nor does the '083 patent's prosecution history, according to Jansen, suggest that the infringer's state of mind is relevant. He also argues that Rapoport does not support the court's view that a direct infringer must purposefully perform the claimed method, and that in any event Rapoport is distinguishable because that case, unlike this case, did not involve a claim to a method of prevention of a disease. According to Jansen, the phrase "a human in need thereof" encompasses a person who does not know that his or her serum levels of folic acid and vitamin B_{12} are adequate. Jansen secondly argues that he presented sufficient evidence of infringement to avoid summary judgment. According to Jansen, Rexall's formulation and labeling are circumstantial evidence of direct infringement by Rexall's customers.

Rexall responds that the court's claim construction does not add an intent element to the claims except as required by the particular language of the claims themselves. Rexall also contends that, just as in Rapoport, the claims in the '083 patent should be interpreted to require that the target group ("human[s] in need thereof") practice the method for the stated purpose ("treating or preventing macrocytic-megaloblastic anemia"), especially where, as here, the prosecution history reveals that both limitations were added for patentability. According to Rexall, a "human in need thereof" is someone either suffering from macrocytic-megaloblastic anemia or at a recognized risk, such as by medical diagnosis, of developing that condition. Rexall also responds that there is no evidence that it markets its product to the target group for the claimed purpose; on the contrary, it contends that it markets its product

only for regulation of blood homocysteine levels. Rexall further contends that, even if there were some evidence of direct infringement by its customers, it is not liable for indirect infringement, for it has not intended to cause infringement and there are substantial noninfringing uses of its product, thereby negating inducement of and contributory infringement.

We begin our claim construction, as always, with the ordinary meaning of the claim language. Rexnord Corp. v. Laitram Corp., 274 F.3d 1336, 1341 (Fed. Cir. 2001). That language requires that the method be performed on "a human in need thereof" and that the method be used "for treating or preventing macrocytic-megaloblastic anemia." The parties do not dispute what "macrocytic-megaloblastic anemia" means; instead, they dispute how the "treating or preventing" phrase and the "to a human in need thereof" phrase should be read. The issue reduces to whether such a human must know that he is in need of either treatment or prevention of that condition.

A similar issue arose in Rapoport, an interference proceeding before the PTO's Board of Patent Appeals and Interferences. The count in that case read as follows:

> A method for treatment of sleep apneas comprising administration of a therapeutically effective amount of a Formula I azapirone compound or a pharmaceutically effective acid addition salt thereof to a patient in need of such treatment . . . .

254 F.3d at 1056 (emphases added). On appeal we gave weight to the ordinary meaning of the preamble phrase "for treatment of sleep apneas," interpreting it to refer to sleep apnea, per se, not just "symptoms associated with sleep apnea." Id. at 1059. Rapoport argued that the count was unpatentable on the ground that a prior art reference disclosed that a form of the compound recited in the claim could be administered, not for treatment of sleep apnea itself, but for treatment of anxiety and breathing difficulty, a symptom of apnea. Id. at 1061. We rejected that argument, stating, "There is no disclosure in the [prior art reference that the

compound] is administered to patients suffering from sleep apnea with the intent to cure the underlying condition." Id. (emphasis added). Thus, the claim was interpreted to require that the method be practiced with the intent to achieve the objective stated in the preamble.

Just as in Rapoport, it is natural to interpret the nearly parallel language in the '083 patent claims in the same way. In both Rapoport and this case, the claim preamble sets forth the objective of the method, and the body of the claim directs that the method be performed on someone "in need." In both cases, the claims' recitation of a patient or a human "in need" gives life and meaning to the preambles' statement of purpose. See Kropa v. Robie, 187 F.2d 150, 152 (CCPA 1951) (stating the rule that a preamble is treated as a limitation if it gives "life and meaning" to the claim). The preamble is therefore not merely a statement of effect that may or may not be desired or appreciated. Rather, it is a statement of the intentional purpose for which the method must be performed. We need not decide whether we would reach the same conclusion if either of the "treating or preventing" phrase or the "to a human in need thereof" phrase was not a part of the claim; together, however, they compel the claim construction arrived at by both the district court and this court.

Our conclusion as to the meaning of the claims is bolstered by an analysis of the prosecution history. The prosecution history is often useful to ascertain the meaning of the claim language. Indeed, claims are not construed in a vacuum, but rather in the context of the intrinsic evidence, viz., the other claims, the specification, and the prosecution history. See DeMarini Sports, Inc. v. Worth, Inc., 239 F.3d 1314, 1327 (Fed. Cir. 2001). In this case, the "treating or preventing macrocytic-megaloblastic anemia" phrase and the "to a human in need thereof" phrase were added to gain allowance of the claims after almost twenty years of repeatedly unsuccessful attempts to gain allowance of claims without those phrases. We must therefore give them weight, for the patentability of the claims hinged upon their presence in the claim language. See Smith v. Magic City Kennel Club, Inc., 282 U.S. 784, 790 (1931)

("The applicant[,] having limited his claim by amendment and accepted a patent, brings himself within the rules that if the claim to a combination be restricted to specified elements, all must be regarded as material, and that limitations imposed by the inventor, especially such as were introduced into an application after it had been persistently rejected, must be strictly construed against the inventor and looked upon as disclaimers."). Furthermore, because both phrases were added simultaneously to overcome the same rejection, they should be read together, meaning that the word "thereof" in the phrase "to a human in need thereof" should be construed to refer to the treatment or prevention of macrocytic-megaloblastic anemia. Finally, that "need" must be recognized and appreciated, for otherwise the added phrases do not carry the meaning that the circumstances of their addition suggest that they carry. In other words, administering the claimed vitamins in the claimed doses for some purpose other than treating or preventing macrocytic-megaloblastic anemia is not practicing the claimed method, because Jansen limited his claims to treatment or prevention of that particular condition in those who need such treatment or prevention. Thus, the '083 patent claims are properly interpreted to mean that the combination of folic acid and vitamin B_{12} must be administered to a human with a recognized need to treat or prevent macrocytic-megaloblastic anemia.

Given that claim construction, we turn to the issue whether Jansen has raised a genuine issue of material fact regarding infringement. We conclude that he has not. Jansen has asserted indirect infringement by Rexall, premised on direct infringement by Rexall's customers. See Met-Coil Sys. Corp. v. Korners Unlimited, Inc., 803 F.2d 684, 687 (Fed. Cir. 1986) ("Absent direct infringement of the patent claims, there can be neither contributory infringement nor inducement of infringement." (citations omitted)). Jansen's theory of infringement is primarily based upon his construction of the claim that those who do not affirmatively know that they do not need to take steps to prevent or treat macrocytic-megaloblastic anemia are still "in need thereof." As explained above, that claim construction

is incorrect. Jansen nonetheless asserts that he has circumstantial evidence of direct infringement by Rexall's customers under the claim construction we and the district court have adopted. Specifically, he contends that Rexall's formulation, having folic acid and vitamin B_{12} in such large quantities as his claims call for, as well as Rexall's labeling stating that "[i]t is especially important to take B-12 along with Folic acid because Folic acid can mask a B-12 deficiency," are evidence that some customers do knowingly take the Rexall product to treat or prevent macrocytic-megaloblastic anemia.

While Jansen is correct that it is theoretically possible that some of Rexall's customers do take the Rexall product knowingly to treat or prevent macrocytic-megaloblastic anemia, and therefore directly infringe his patent, his evidence is quite weak. In fact, he has shown no more than a theoretical possibility or "metaphysical doubt," which is insufficient to create a genuine issue of material fact. See Anderson, 477 U.S. at 261 (citing Matsushita Elec. Indus. Co. v. Zenith Radio Corp., 475 U.S. 574, 586 (1986)). The district court's decision that there were no genuine issues of material fact on the question of infringement was therefore correct.

Use of an over-the-counter product like Rexall's is quite different from the use of a product pursuant to a prescription from a medical doctor. In the latter case, a prescription is evidence of a diagnosis and a knowing need to use the product for the stated purpose. Jansen does not have evidence of that in this case. Rexall's product is provided with a label stating that the product can be used for maintenance of blood homocysteine levels, and purchasers do not necessarily know that they are in need of preventing or treating macrocytic-megaloblastic anemia. Instead, Jansen has only conjecture that some purchasers of the Rexall product might meet the daim requirements. The district court therefore did not err in holding that he failed to present sufficient proof of infringement to create a genuine issue of material fact and to thereby avoid summary judgment of noninfringement.

CONCLUSION

The district court correctly construed the claims of the '083 patent and properly determined that Jansen did not present sufficient evidence to create a genuine issue of material fact relating to infringement by Rexall. Accordingly, we

AFFIRM.

ATTACHMENT AD

Control of Melanosome Transfer by Promoting Shrinkage or Expansion of Melanocyte Dendrites

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Abstract

Melanosomes synthesized within melanocytes are transferred to keratinocytes through melanocyte dendrites, resulting in a constant supply of melanin to the epidermis which determines skin pigmentation. Theoretically, if we can find an effective way to control this supply of melanin to the epidermis, skin color could be darkened or lightened. The objective of this study was to find safe and effective methods to inhibit or promote melanosome transfer by the shrinkage or expansion of melanocyte dendrites.

Methylophiopogonanone B and centaureidin inhibited melanosome transfer to keratinocytes as well as melanocyte dendrite outgrowth. Methylswertianin and comfrey extract promoted not only melanosome transfer to keratinocytes but also expansion of melanocyte dendrites.

Methylophiopogonanone B and centaureidin suppressed pigmentation in a three-dimensional skin culture model through the inhibition of melanocyte dendrite outgrowth. Methylswertianin and comfrey extract activated pigmentation in a three-dimensional skin culture model by expansion of melanocyte dendrites.

Our experimental findings suggest the possibility of manipulating human skin color by controlling melanosome transfer to cause shrinkage or expansion of dendrites. A combination of effective agents, in addition to the ones identified in this work, could result in the creation of very unique cosmetic products that would precisely control the darkening or lightening of skin tone.

Keywords: Methylophiopogonanone B, centaureidin, methylswertianin, comfrey extract, melanocyte dendrites

Paper presented at the 23rd IFSCC Congress 2004, Orlando, Florida, USA

INTRODUCTION

The popularity of self-tanning products in many Western nations and whitening products in Asian countries is evidence of the persistent human desire to control skin color. However, there are many difficulties with the products currently available for such purposes. For example, dihydroxyacetone-based tanning products do not always produce the desired color [1], and whitening ingredients, including many tyrosinase inhibitors such as hydroquinone and kojic acid, are ineffective at low concentrations and unsafe at high concentrations [2-4]. Thus, there is a need for better skin tone-altering products. The epidermal melanin unit consists of the symbiotic interaction between melanocytes and an associated pool of keratinocytes. It has been estimated that a single melanocyte interacts with approximately 36 keratinocytes in the basal and suprabasal layers of the epidermis [5]. Melanosomes synthesized within melanocytes are transferred to keratinocytes through melanocyte dendrites. The dendrites of melanocytes transfer melanin to surrounding keratinocytes in response to hormones such as melanocyte stimulating hormone and ultraviolet light, both of which stimulate melanin synthesis and melanosome transfer [6, 7]. During this process dendrite extension is necessary for melanosome transfer to keratinocytes because melanocytes constitute a minor population in the epidermis and must supply melanin to many keratinocytes [8]. This results in a constant supply of melanin reaching the epidermis. In this way, skin pigmentation is determined. Theoretically, skin color could be darkened or lightened by controlling the supply of melanin reaching the epidermis. One possible way of controlling this supply is to promote shrinkage or expansion of dendrites. The objective of this study was to find safe and effective methods that would inhibit or promote melanosome transfer by shrinking or expanding the melanocyte dendrites. After examining many different agents for this purpose, we focused our investigation on botanical extracts since they are known to contain a large number of chemicals and to have a relatively low cytotoxicity. Thus, by screening a large number of selected extracts, promising active ingredients that would be safe and effective for our purpose could be identified.

EXPERIMENTAL

Cells and cell culture

Normal human epidermal melanocytes and normal human epidermal keratino-

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cytes were obtained from Kurabo Biomedical Business (Osaka, Japan). Normal human epidermal melanocytes were main-tained in melanocyte complete 154S culture medium supplemented with 0.5% fetal bovine serum, 5 µg/mL insulin, 0.5 µM hydrocortisone, 5 µg/mL transferrin, 3 µg/mL heparin, 3 ng/mL human basic fibroblast growth factor, 10 ng/mL phorbol-12-myristate-13-acetate and 0.2% bovine pituitary extract (all purchased from Kurabo). Normal human epidermal keratinocytes were maintained in keratinocyte complete 154S culture medium supplemented with 5 µg/mL insulin, 0.5 µM hydrocortisone, 5 µg/mL transferrin, 200 pg/mL human recombinant epithelial growth factor, and 0.2% bovine pituitary extract (all purchased from Kurabo).

Assay of inhibition of melanocyte dendrite outgrowth

Normal human epidermal melanocytes were plated on a 48-well microtiter plate at a density of 3,000 cells per well and treated 24 h later with a plant extract for a total of 48 h. Melanocyte morphology was observed microscopically 24 h and 48 h after treatment. When melanocyte dendrite shrinkage was observed, we confirmed that the shrinkage was not due to cell toxicity by removing the plant extract from the culture medium and observing the melanocytes.

Assay of expansion of melanocyte dendrites

Normal human epidermal melanocytes were maintained in a culture medium lacking phorbol-12-myristate-13-acetate and bovine pituitary extract and then plated on a 48-well microtiter plate at a density of 3,000 cells per well. After 24 h incubation, plant extracts were added to each well. The microtiter plate was incubated for 48 h and melanocyte morphology was observed.

Isolation of methylophiopogonanone B from ophiopogon tuber

Methylophiopogonanone B (5,7-dihydroxy-6,8-dimethyl-3-(4-methoxybenzyl)-chroman-4-one) and methylophiopogonanone A (5,7-dihydroxy-6,8-dimethyl-3-(3,4-methylenedioxybenzyl)-chroman-4-one) were isolated from *Ophiopogon japonicus* as follows. Methanol extracts of ophiopogon tuber were evaporated to dryness *in vacuo*. The residue was dissolved in water, extracted with ethyl acetate and concentrated under reduced pressure. The residue was eluted through a column of silica gel with chloroform to give a mixture of methylophiopogonanone A and B. The mixture was purified by Sephadex LH-20 column chromatography using methanol as the eluent. Methylophiopogonanone B was identified by ^{1}H-NMR and ^{13}C-NMR, the results of which were in agreement with previously published data [9].

Isolation of centaureidin from Achillea millefolium

Centaureidin (5,7,3'-trihydroxy-3,8,4'-trimethoxyflavone) was isolated from *Achillea millefolium*. The leaves were extracted with methanol and concentrated *in vacuo*. The residue was poured into water and extracted with ethyl acetate. The organic layer was concentrated under reduced pressure, and the residue was purified by silica gel chromatography using chloroform as the eluent to give pure centaureidin that was subsequently crystallized from chloroform. Analyses by ^{1}H-NMR, ^{13}C-NMR, EI-MS, and X-ray crystallography proved that our extract was in fact centaureidin, as our results were in agreement with previously published data [10].

Isolation of methylswertianin from the swertia herb

Methylswertianin (1,8-dihydroxy-3,7-dimethoxyxanthone) was isolated from the swertia herb. The leaves were extracted with methanol and the solvent evaporated under reduced pressure. The extract was suspended in distilled water and extracted with ethyl acetate. The ethyl acetate layer was concentrated under reduced pressure and then subjected to silica gel column chromatography and elution with chloroform to yield a crude fraction that included methylswertianin. This fraction was repeatedly purified by silica gel chromatography using chloroform as the eluent to obtain pure methylswertianin. Methylswertianin was identified by ^{1}H-NMR and ^{13}C-NMR, the results of which were in agreement with previously published data [11].

Preparation of comfrey extract

Comfrey was extracted with methanol and concentrated under reduced pressure to produce a dark green viscous mass which was subjected to silica gel column chromatography. The column was developed with 0%, 5%, 10%, 20%, and 30% methanol in chloroform to obtain the various fractions. All these fractions were tested for their ability to expand melanocyte dendrites; only the 30% methanol in chloroform eluted fraction showed potent activity.

Cytotoxicity of methylophiopogonanone B and centaureidin in normal human epidermal melanocytes

Cytotoxicity was assessed using a commercially available kit (Cell Counting Kit-8; Dojindo Co. Kumamoto, Japan) containing the highly water soluble tetrazolium salt, WST-8, (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Normal human epidermal melanocytes were plated on 96-well microtiter plates at a density of 10,000 cells per cm^2. After 24 or 48 h in the presence of various concentrations of methylophiopogonanone B or centaureidin, cells were assayed according to the manufacture's protocol and incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (Benchmark Plus; BioRad, Hercules, CA).

Measurement of melanin production

This part of the experiment was done according to the method described by Whittaker [12]. Briefly, normal human epidermal melanocytes were incubated with 0.0185 MBq [^{14}C] thiouracil and 1 µM methylophiopogonanone B or 0.5 µM centaureidin for 5 days, then trypsinized and precipitated by centrifugation. Next, the pelleted cells were precipitated with 10% trichloroacetic acid and then lysed in scintillation cocktail. Radioactivity was measured using a liquid scintillation counter (LSC6100; ALOKA, Tokyo, Japan). The calculated amount of radioactivity was equilibrated using the viable cells ratio from the WST-8 assay.

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Scanning laser confocal microscopy

Cells stained with the succinimidyl ester of carboxy fluorescein diacetate (CFDA) (Molecular Probes, Eugene, Oregon) were scanned according to the procedure described by Minwalla *et al.* [13]. Briefly, normal human epidermal melanocytes stained with 2 µM CFDA were co-cultured with normal human epidermal keratinocytes in a ratio of 1:2. For various periods of time the co-cultures were maintained in complete melanocyte medium and complete keratinocyte culture medium in a ratio of 1:2. The cells were then fixed with 4% formalin in phosphate-buffered saline and mounted with fluoromount G (Southern Biotechnology Associates Inc., Birmingham, Alabama). The CFDA transferred from the melanocytes to the keratinocytes was observed using an Olympus BX51 laser microscope (Olympus, Tokyo, Japan).

Histochemistry of reconstructed epidermis

The reconstructed human epidermis (Kurabo) consisted of normal human-derived epidermal keratinocytes and melanocytes that had been cultured to form a multilayered, highly differentiated model. Reconstructed epidermis was incubated in medium containing the specific agents being tested. The medium and agents were replaced every other day. After treatment the epidermis sheets were prepared, and skin sections were processed for histochemical Fontana Masson staining.

RESULTS AND DISCUSSION

Shrinkage of normal human melanocyte dendrites

Effects of methylophiopogonanone B and centaureidin on normal human epidermal melanocytes morphology

From among the large number of plant extracts that we screened, ophiopogon tuber and *Achillea millefolium* extracts were found to be most effective in shrinking normal human melanocyte dendrites. Further investigation revealed that methylophiopogonanone B, a component of the ophiopogon tuber extract, and centaureidin, a component of the *Achillea millefolium* extract ***(Figure 1)***, were responsible for the observed effects ***(Figure 2)***. We ascertained that the shrinkage was not due to cell toxicity because removal of methylophiopogonanone B and centaureidin from the culture medium after 48 hours of treatment resulted in the elongation of the melanocyte dendrites to a normal state without any apparent damage 24 h later ***(Figure 2H and L)***.

Cytotoxicity of methylophiopogonanone B and centaureidin in normal human epidermal melanocytes

We examined the viability of normal human epidermal melanocytes monocultures after exposure to various concentrations of methylophiopogonanone B and centaureidin. Methylophiopogonanone B and centaureidin shrank dendrites at concentrations of 1 µM and 0.5 µM, respectively. Methylophiopogonanone B (1 µM)

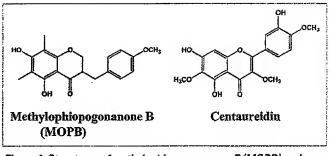

Figure 1: *Structures of methylophiopogonanone B (MOPB) and centaureidin, both of which cause shrinkage of normal human melanocyte dendrites*

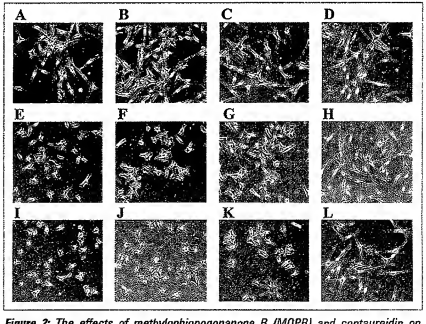

Figure 2: *The effects of methylophiopogonanone B (MOPB) and centaureidin on melanocyte morphology. A, B, C, and D treated with DMSO blank, (A) 24 h treatment, (B) 48 h treatment, (C) 72 h treatment, (D) 24 h after removing DMSO from the medium after 48 h treatment. E, F, G, and H treated with 1 µM methylophiopogonanone B in DMSO, (E) 24 h treatment, (F) 48 h treatment, (G) 72 h treatment, (H) 24 h after removing methylophiopogonanone B from the medium after 48 h treatment. I, J, K, and L treated with 0.5 µM centaureidin in DMSO, (I) 24 h treatment, (J) 48 h treatment, (K) 72 h treatment, (L) 24 h after removing centaureidin from the medium after 48 hours of treatment.*

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and centaureidin (0.5 µM) showed no toxicity to the normal human melanocytes based on the WST-8 assay method ***(Figure 3)***. Thus, the dendrite retraction induced by methylophiopogonanone B and centaureidin appeared to be reversible and not associated with toxicity. Methylophiopogonanone B and centaureidin would be safe and effective for shrinkage of normal human epidermal melanocytes.

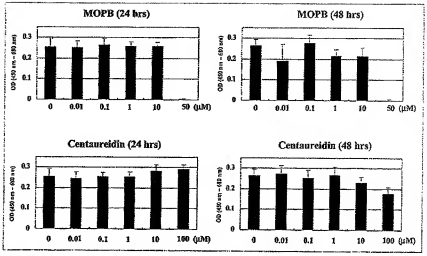

Figure 3: *Cytotoxicity of methylophiopogonanone B (MOPB) and centaureidin in normal human epidermal melanocytes. Cell viability was examined at 24 and 48 h after culture at the indicated concentrations of methylophiopogonanone B and centaureidin. Each value represents the mean OD 450 nm minus OD 680 nm of 6 determinations ± standard deviation.*

Effects of methylophiopogonanone B and centaureidin on melanin synthesis

The dynamic changes in melanocyte cell morphology should have an influence on the basic function of melanocytes, including melanogenesis. Melanin synthesis was assessed in normal human epidermal melanocytes after exposure to methylophiopogonanone B (1 µM) or centaureidin (0.5 µM) for 5 days. Melanin synthesis was reduced slightly by dendrite shrinkage with the addition of methylophiopogonanone B and centaureidin ***(Figure 4)***.

Effects of methylophiopogonanone B and centaureidin on inhibition of melanosome transfer due to dendrite shrinkage

Melanocytes transfer melanosomes through their dendrites to surrounding keratinocytes in the skin. To study the effects of methylophiopogonanone B and centaureidin on melanosome transfer, co-cultures of normal human melanocytes and keratinocytes were used to test methylophiopogonanone B and centaureidin for their ability to reduce melanosome transfer by shrinking the dendrites. The transfer of fluorochrome from labeled melanocytes to keratinocytes in an *in vitro* model system has been observed [13]. CFDA-labeled melanocytes were co-cultured with keratinocytes in the presence of methylophiopogonanone B (1 µM) or centaureidin (0.5 µM) for 5 days. The DMSO-treated cells were almost all keratinocytes that contained fluorochrome obtained from surrounding melanocytes ***(Figure 5D)***. Methylophiopogonanone B and centaureidin effectively inhibited the transfer of fluorochrome to the keratinocytes by causing dendrite shrinkage ***(Figure 5E and 5F)***. Our results demonstrated that melanosome transfer can be interrupted *in vitro* by adding methylophiopogonanone B or centaureidin to co-cultures of melanocytes and keratinocytes. Hakozaki *et al.* reported that niacinamide (vitamin B_3) suppresses melanosome transfer, and in their human clinical study topical application of niacinamide produced a remarkable reduction of cutaneous pigmentation, including moderate lentigo senilis melasma and freckles [14], suggesting that a drug which inhibits melanosome transfer could be a breakthrough agent for reducing melanin in the epidermis. Cells treated with niacinamide (1 mM) transferred a small amount of fluorochrome to keratinocytes (data not shown). The inhibition of melano-some transfer by niacinamide was reported to be approximately 35-68% [14]. On the other hand, melanocytes treated with methylophiopogonanone B or centaureidin did not transfer fluorochrome to keratinocytes, and all of the dye remained in the melanocytes ***(Figure 5E and F)***. Thus, methylophiopogonanone B and centaureidin appeared to block melanosome transfer completely, whereas niacinamide did so only partially.

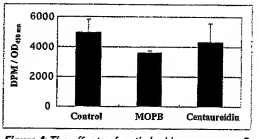

Figure 4: *The effects of methylophiopogonanone B (MOPB) and centaureidin on melanin synthesis. Melanin production and cell viability were assessed after 5 days in the presence of methylophiopogonanone B (1 µM) or centaureidin (0.5 µM). Each value represents the mean amount of radioactivity per viable cell of 3 determinations ± standard deviation.*

Effects of methylophiopogonanone B and centaureidin on melanocytes maintained in a three-dimensional culture model

Further studies were performed on the effects of methylophiopogonanone B and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment the reconstructed epidermis was observed for melanocyte morphology and melanin distribution. To study melanocyte morphology, the prepared epidermis sheets were processed for Fontana Masson staining. Based on ultrastructure studies, methylophiopogo-

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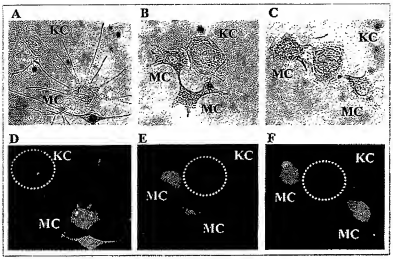

Figure 5: The effects of methylophiopogonanone B (MOPB) and centaureidin on inhibition of melanosome transfer due to dendrite shrinkage – confocal images of co-cultures of CFDA-labeled melanocytes (MC) with keratinocytes (KC). Differential interference contrast images are shown above each confocal image to depict the interaction between the melano-cytes and keratinocytes. A and D treated with DMSO blank for 5 days, B and E treated with 1 µM methylophiopogonanone B in DMSO for 5 days, C and F treated with 0.5 µM centaureidin in DMSO for 5 days.

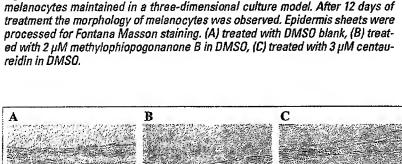

Figure 6: The effects of methylophiopogonanone B (MOPB) and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment the morphology of melanocytes was observed. Epidermis sheets were processed for Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 2 µM methylophiopogonanone B in DMSO, (C) treated with 3 µM centaureidin in DMSO.

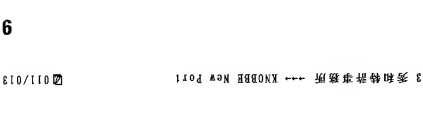

Figure 7: Effects of methylophiopogonanone B (MOPB) and centaureidin on melanocytes maintained in a three-dimensional culture model. After 12 days of treatment cultured skin sections were processed for histochemical Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 2 µM methylophiopogonanone B in DMSO, (C) treated with 3 µM centaureidin in DMSO.

nanone B and centaureidin were found to induce shrinkage of melanocyte dendrites ***(Figure 6)***. To study melanin distribution, cultured skin sections were processed for histochemical Fontana Masson staining. Methylophiopogonanone B and centaureidin were shown to clearly inhibit melanosome transfer to keratinocytes through dendrite shrinkage in the three-dimensional culture model ***(Figure 7)***.

Expansion of normal human melanocyte dendrites

Identification of methylswertianin

Encouraged by the discovery of the plant extracts which could effectively shrink melanocyte dendrites, we decided to search for extracts which would have the opposite effect, namely expanding dendrites to produce more melanin. After screening a large number of extracts for this purpose, we discovered that swertia herb and comfrey extracts are the most effective. Further evaluation revealed that methylswertianin ***(Figure 8)***, a component of swertia herb extract, was responsible for the observed effects of the swertia herb extract.

Effects of methylswertianin and comfrey extract on the promotion of melanosome transfer due to dendrite expansion

To study the effects of methylswertianin and comfrey extract on melanosome transfer, co-cultures of normal human melanocytes and keratinocytes were used to test methylswertianin and comfrey extract for the promotion of melanosome transfer due to dendrite expansion. CFDA-labeled melanocytes were co-cultured with keratinocytes in the presence of methylswertianin or comfrey extract. Methylswertianin and comfrey extract effectively increased the transfer of the fluorochrome to keratinocytes due to dendrite expansion ***(Figure 9)***. Our results demonstrated that melanosome transfer can be promoted *in vitro* by adding methylswertianin or comfrey extract to co-cultures of melanocytes and keratinocytes. Seiberg *et al.* reported that the protease-activated receptor 2 (PAR-2) expressed on keratinocytes, but not on melanocytes, is involved in melanosome transfer and

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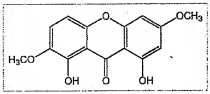

Figure 8: Structure of methylswertianin, which causes expansion of normal human epidermal melanocytes dendrites.

therefore may regulate pigmentation. They suggested the regulation of pigmentation, mediated by the activation or inhibition of the keratinocyte receptor PAR-2 [15]. As our work focused on the melanocyte dendrites, we did not demonstrate that the PAR-2 expressed or suppressed on keratinocytes by adding methylophiopogonanone B, centaureidin, methylswertianin or comfrey extract. We also did not investigate whether these agents affected other dendritic cells such as Langerhans cell.

Effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model

Further studies were performed on the effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model. After 9 days of treatment, epidermis sheets were processed for Fontana Masson staining. Based on ultrastructure studies, methylswertianin and comfrey extract were found to induce expansion of melanocyte dendrites ***(Figure 10)***.

CONCLUSION

Our experimental findings suggest the possibility of manipulating human skin color by controlling melanosome transfer through the shrinkage or expansion of dendrites. Our work involved the use of human melanocytes and keratinocytes. We found that methylophiopogonanone B and centaureidin, active components present in ophiopogon tuber and *Achillea millefolium* extract, can induce shrinkage of melanocytes, thus causing a reduction in melanosome transfer to keratinocytes, which suppresses skin pigmentation. Furthermore, we found that methylswertianin and comfrey extract have the opposite effect by promoting the expansion of melanocyte dendrites, thereby increasing melanosome transfer, which increases skin pigmentation.

We focused our investigation on botanical extracts since they are known to have a relative low cytotoxicity and to be distributed all over world. Both chemicals and plant extracts including active materials produced the desired effects such as shrinkage or expansion of melanocyte dendrites. A combination of effective agents, in addition to the ones identified in this work, could result in the creation of very unique cosmetic products that precisely control the darkening or lightening of skin tone.

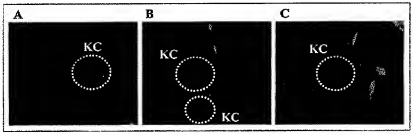

Figure 9: The effects of methylswertianin and comfrey extract on the promotion of melanosome transfer due to dendrite expansion – confocal images of co-cultures of CFDA-labeled melanocytes with keratinocytes (KC). (A) treated with DMSO blank for 6 days, (B) treated with 0.2 mM methylswertianin in DMSO for 6 days, (C) treated with 0.001% comfrey extract in DMSO for 6 days.

Figure 10: The effects of methylswertianin and comfrey extract on melanocytes maintained in a three-dimensional culture model. After 9 days of treatment melanocyte morphology was examined. Epidermis sheets were processed for Fontana Masson staining. (A) treated with DMSO blank, (B) treated with 0.4 mM methylswertianin in DMSO, (C) treated with 0.001% comfrey extract in DMSO.

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